Ecotoxicology and Environmental Safety 163 (2018) 37–46

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Physiological and behavioural responses to acid and osmotic stress and effects of *Mucuna* extract in Guppies

Mahammed Moniruzzaman[a,1], Joyita Mukherjee[b,1], Lisa Jacquin[c], Debosree Mukherjee[a], Pubali Mitra[a], Santanu Ray[d], Suman Bhusan Chakraborty[a,*]

[a] Fish Endocrinology Research Unit, Department of Zoology, University of Calcutta, Kolkata 700019, India
[b] Department of Zoology, Krishna Chandra College, University of Burdwan, Hetampur, Birbhum 731124, West Bengal, India
[c] Laboratoire Evolution & Diversité Biologique EDB, UMR 5174, Université de Toulouse, UPS, CNRS, IRD, 118 route de Narbonne, 31062 Toulouse, France
[d] Ecological Modeling Laboratory, Department of Zoology, Visva-Bharati University, Santiniketan 731235, India

ARTICLE INFO

*Keywords:*
Stress response
Salinity
pH
Plant extract
Cortisol
Biomarkers

ABSTRACT

Variation in pH (acidification) and salinity conditions have severe impact at different levels of biological organization in fish. Present study focused to assess the effects of acidification and salinity changes on physiological stress responses at three different levels of function: i) hormonal and oxidative response, ii) osmoregulation and iii) reproduction, in order to identify relevant biomarkers. Second objective of the study was to evaluate the efficacy of plant (*Mucuna pruriens*) extract for alleviating pH and salinity related stress. Guppies (*Poecilia reticulata)* were exposed to different pH (6.0, 5.5, 5.0) and salinity (1.5, 3.0, 4.5 ppt) for 7, 14 and 21 days. Following exposure to stress for respective duration, fish were fed diet containing methanol extract of *Mucuna* seeds (dose 0.80 gm/kg feed) for 7, 14 and 21 days to measure their possible recovery response. Stress hormone (cortisol), hepatic oxidative stress parameters [superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd), glutathione peroxidise (GPx), glutathione S-transferase (GST), malondialdehyde (MDA), glutathione (GSH)], gill osmoregulatory response ($Na^+$-$K^+$ATPase activity), sex steroid profiles and mating behaviours (gonopodial thrust and gestation period) were estimated. Cortisol and MDA levels increased with dose and duration of acid and salinity stress, and cortisol levels were higher in males than in females. Effect on $Na^+$-$K^+$ATPase activity was more intense by salinity stress rather than pH induced stress. Both acid and salinity stress reduced sex steroid levels, and mating response was highly affected by both stresses in a dose- and duration-dependent manner. *Mucuna* treatment reduced stress-induced alteration of cortisol, MDA, $Na^+$-$K^+$ATPase activity and reproductive parameters. Dietary administration of *Mucuna* seed extract decreased the intensity of environmental stressors at all three functional levels. *Mucuna* treatment was more effective against salinity stress than acid stress. Thus, cortisol, oxidative stress marker MDA and $Na^+$-$K^+$ATPase could be effective indicators for acid and salinity stress in wild and domestic fish populations. Dietary administration of *Mucuna* extract may limit the detrimental effects of acidification and salinity variations that are the inevitable outcomes expected under global climate change conditions.

## 1. Introduction

Pollution and temperature changes are increasingly exposing aquatic systems to water acidification and salinity alterations throughout the world (Guinotte and Fabry, 2008; Feely et al., 2010; Vaz et al., 2015). Such environmental stressors are affecting wild and farmed fish raised in semi-natural conditions, which may result in severe loss of biodiversity and cause economic losses in the near future (Williams and Rota, 2010). However, the physiological and reproductive responses of freshwater fish to such stressors are still poorly understood.

Most fish species survive in a narrow range of pH and salinity. Any deviation from the optimal range of pH or salinity or both can disrupt their physiological functions (Whitney et al., 2016; Mubarik et al., 2015; Serrano et al., 2010), eventually, alter their life history traits such as growth (Ong et al., 2015) and reproduction (Kwong et al., 2014). Recent studies helped deciphering information on the hyper-hypo-osmoregulators (Kelly et al., 2007), and the physiological stress responses

* Correspondence to: Department of Zoology, Ballygunge Science College, University of Calcutta, Kolkata 700019, West Bengal, India.
*E-mail address:* sumanbc76@gmail.com (S.B. Chakraborty).
[1] Authors have contributed equally.

https://doi.org/10.1016/j.ecoenv.2018.07.053
Received 22 March 2018; Received in revised form 11 July 2018; Accepted 13 July 2018
Available online 19 July 2018
0147-6513/ 

to pH and salinity changes (Katoh et al., 2008; Scott et al., 2008; Tipsmark and Madsen, 2009). Exposure to suboptimal pH and salinity levels leads to a rapid alteration in the cascade of molecular and physiological responses (Makrinos and Bowden, 2016; Xu et al., 2015). The primary responses involve hormonal parameters (cortisol secretion), secondary responses include oxidative stress responses (different biomarkers of oxidative stress and damages) and osmotic regulation responses ($Na^+$-$K^+$ATPase activity), followed by tertiary response such as reproductive changes (Xu et al., 2015).

During the primary response, cortisol mediates a range of physiological and behavioural responses against a variety of environmental stressors (Pottinger, 2017). Some of these changes can promote survival through increased metabolism and detoxification processes. The secondary response to suboptimal pH or salinity may be enhanced free radicals causing oxidative damages (Sies, 2016). Exposure to acute and/or chronic stressors can increase oxidative stress and result in irreparable damages to cell membranes, inactivation of several vital enzymes through the alteration of different transcription factor (Moniruzzaman et al., 2018). The effective control and rapid elimination of reactive oxygen species (ROS) is essential to the proper functioning, survival and reproduction of the aquatic organisms.

One of the major challenges to improve our understanding of the stress responses of fish to these stressors is to identify simple and reliable biomarkers of oxidative stress in fish (Lushchak, 2011; Mukherjee et al., 2017a, 2017b). Enzymes involved in antioxidant defences include radical scavenging enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Klein et al., 2017; Halliwell and Gutteridge, 2006). Small water-soluble antioxidant compounds such as glutathione (GSH) is also one of the most reliable free radical scavenger of cells (Birben et al., 2012). Lipid peroxidation and malondialdehyde (MDA) levels are a direct reflection of the amount of oxidative damages as well. The activity of the membrane-bound enzyme $Na^+$-$K^+$ATPase changes accordingly with the amount of lipid peroxidation (Babu et al., 2006). This ultimately alters membrane function and permeability that may lead to destruction of cells or whole cell systems (Rind et al., 2017). CAT, SOD, GPX, GSH, MDA and $Na^+$-$K^+$ATPase activities are thus good potential biomarkers of saline and pH stressors but experimental studies comparing their relative intensity of response at different stress doses are still rare.

Environmental stressors can also affect fish reproduction (tertiary response). pH and salinity stress have been found to affect sperm and egg maturation, reproductive development, egg survival and fertilization in different aquatic species (Miller et al., 2015; Dadras et al., 2017; Allen et al., 2017). Alterations in different reproductive attribute may thus be important biomarkers for such environmental stress in fish. In this study, we performed a comprehensive characterization of physiological responses at different functional levels and aimed at identifying significant biomarkers regarding the effects of pH and salinity using an experimental approach in controlled conditions.

Some antioxidant molecules are known to have positive effects in alleviating these stress responses. Plant extracts in the dietary supplements have gained interest recently due to their nutritional values and positive effects on growth and reproductive performance in many fish species (Chakraborty and Hancz, 2011; Chakraborty et al., 2014). Medicinal plant *Mucuna pruriens* contains high amounts of proteins and carbohydrates and is a rich source of macro- and microelements. Alcoholic extracts of *Mucuna* seeds were shown to have potential antioxidant activity to regulate stress-induced lipid peroxidation (Madhyastha et al., 2011). Different preparations of *Mucuna* were recently used for the management of several free radical-mediated diseases (Rai et al., 2017). However, the effects of *M. pruriens* on stress responses antioxidant enzymes and reproduction in fish in response to environmental pH and salinity stress are not yet documented anywhere.

To test the effects of saline and pH stressors on the stress response of fish at different biological levels and the effects of *Mucuna* in modulating such stress responses, we used the guppy, *Poecilia reticulata* as a model species. *P. reticulata* is a popular freshwater species with high ornamental value and is an important biological tool to study the reproductive physiology throughout the world (Kavitha and Subramanian, 2011), because of its viviparity and short reproductive period (Guevara-Fiore and Endler, 2018). Though they live in almost every freshwater body near the coastal fringes, guppies have low tolerance to brackish water and found to colonize low salinity brackish habitats. In the wild and in captive conditions, their optimal pH is 6.5–8.0 and optimal salinity levels are about 2 ppt but beyond 3 ppt the survival rate decreases significantly (Kavitha and Subramanian, 2011).

The first objective of the study was to test the physiological consequences of pH and salinity stressors on guppy responses at three functional levels. First, we recorded the alterations in cortisol secretion (hormonal response). Second, we recorded the status of oxidative stress in the hepatic tissue (oxidative response), as well as the $Na^+$-$K^+$ATPase activity in gill (osmoregulatory response). Third, we recorded the reproductive response by measuring sex steroid profiles and mating behaviour (gonopodial thrust in males and gestation period in females) to determine the impacts of altered pH and salinity and identify the central biomarkers of eco-physiological stress. The second objective of the study was to evaluate the efficacy of the *Mucuna* plant extracts for alleviating pH and salinity stress at these three biological levels in guppies to assess the efficacy of *Mucuna* treatment to maintain the biomarkers at equilibrium.

## 2. Materials and methods

### 2.1. Model species and acclimation

Guppies were purchased from the local market and kept into large sized tank (180 × 90 × 60 cm) for ten days for acclimatization. During the acclimatization period, fish were fed an artificial diet containing 30% crude proteins (Tetra Bits Complete, Tetra). Throughout the entire period of acclimatization and experiment, all fish were maintained under constant temperature (T = 27 ± 0.5 °C), similar photoperiod (14 L: 10 D), optimum hardness (8.5 ± 0.8) and dissolved $O_2$ (6.5 ± 0.5 mg/L). Salinity ranges (0.1 ± 0.05) were also checked for all the fish during the acclimatization period and for control fish throughout the experiment. Similarly, the pH ranged between 6.7 and 6.9 for all the fish during acclimatization period and throughout the experiment schedule for control fish. During the entire experimental duration, levels of ammonia (< 4 ppm), nitrate (< 30 ppm), and nitrite (< 1.0 ppm) were measured daily using aquarium test kits. If levels exceeded the aforementioned limits, a 50% water change was performed. Otherwise, 25% of the water was changed every other day. Detritus were removed from the experimental systems through siphoning. Proper care was taken to avoid any sudden changes in temperature, salinity and pH during acclimatization period. No mortality was observed during the entire course of the experiment.

### 2.2. Experimental design

After 10 days of acclimatization, fish were divided into two categories: experimental and control. Experimental fish were again subdivided into two equal groups. One experimental group was exposed to pH stress and the other one to salinity stress (Fig. 1). The control category was not exposed to any pH or salinity stress. The experiment was conducted in static water systems. Salinity and pH were measured daily and adjusted as required both in control and experimental groups. Such adjustments were conducted in stock water prior to addition to the experimental aquaria to minimize stress to the fish.

Tap water was first provided into a mixing tank in which the pH was regulated by the addition of 10% sulphuric acid using a peristaltic pump controlled by an automatic pH controller. The experimental aquaria (90 × 60 × 60 cm) were filled with this pH-adjusted water (± 0.05 the desired pH) from the mixing tank. Fish were exposed to

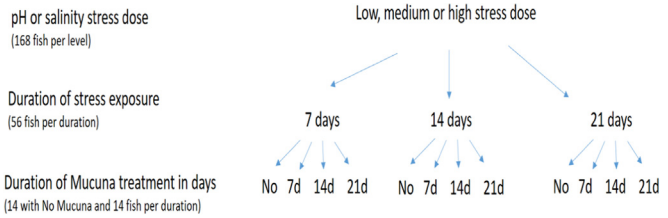


**Fig. 1.** Experimental design with three different dose of pH stress (low: pH 6.0, medium: pH 5.5, high: 5.0, 168 fish each) or salinity stress (low: 1.5 ppt, medium: 3.0 ppt, high: 4.5 ppt, 168 fish each) for different durations of stress exposure (7, 14 and 21 days, 56 fish each). Treatment with *Mucuna* was applied after stress exposure for different durations (No *Mucuna*, *Mucuna* for 7days, *Mucuna* for 14 days or *Mucuna* for 21 days, 14 fish each). In all groups, 8 fish were used for physiological measurements and 6 fish were used for behavioural measurements (see methods). In addition, 42 control fish were kept without any stress or treatment (not shown). In each group, half the fish were females and half were males. The entire experimental set up was conducted in 3 replicates.

three different doses of pH stressor (low dose of stressor: pH 6.0, medium: pH 5.5 and high: pH 5.0, 168 fish per level) for three different durations (7, 14 and 21 days, 56 fish per duration) (Fig. 1). For salinity stress, fish were similarly exposed to three different salinity doses (low saline stress dose: 1.5 ppt, medium: 3.0 ppt and high: 4.5 ppt, 168 fish per level) and each of the doses was continued for three different durations (7, 14 and 21 days, 56 fish per duration) (Fig. 1).

Control fish (42 in number) were maintained in tap water with an optimal salinity (0.1 ± 0.05 ppt) and nearly neutral pH (6.8). In each group, half the fish were females and half were males. The entire experimental set up was conducted in three replicates. During this period of stress experiment, both control and experimental fish were fed an artificial diet containing 30% crude proteins (Tetra Bits Complete, Tetra).

### 2.3. Mucuna seed treatment

*Mucuna* seeds were obtained from a local plant market, washed in sterile distilled water, air-dried and powdered carefully. Powdered plant materials (250 gm) were extracted with 500 ml methanol in a Soxhlet apparatus and the extracts were evaporated to dryness under pressure at 45 °C using rotary evaporator and stored under nitrogen at − 20 °C in amber glass bottle.

After their respective duration of pH/salinity stress exposure (7, 14, 21 days), fish from each experimental group were subsequently fed diet containing methanol extract of *Mucuna* seed (dose 0.80 gm/kg feed) for different time schedules (another 7, 14 and 21 days, 14 fish per duration) to measure their possible recovery response (Fig. 1). During this recovery period, control fish were fed diet containing no plant extract.

Plant extracts were dissolved in dimethyl sulfoxide (DMSO) and added to finely ground (< 500–1000 μm) artificial diet (Tetra Bits Complete, Tetra) (Moundipa et al., 2005). The feed was then wetted with deionized water, mixed thoroughly, formed into pellets with a pelleter (diameter 2 mm), and dried at room temperature. Pelleted feed was pulverized before feeding to the fish.

### 2.4. Collection of tissue samples

Immediately after completion of each experiment schedule (at day 7, 14, 21, 28, 35 and 42) fish (n = 12 females, 12 males) from experimental groups were anesthetized with phenoxy-ethanol (1: 20,000, v/v) and sacrificed 15 min after their capture (Fig. 1). Control fish (n = 12 females, 12 males) were sacrificed at the beginning of the experiment (at day 0), after 21 days of stress experiment (at day 21), and after 21 days of recovery period (at day 42). Control fish showed no significant variations in any of the measured parameters at all these times. Rapid dissection was followed for the collection of gill and liver tissues, and serum from each fish. For hormone assay, the samples were extracted from the serum using methanol as solvent. Serum was collected in 12 × 15 ml EIA tube, methanol was added at 1:3 ratio to it and vortexed for mixing. After centrifugation (at 2000 rpm for 15 min), the supernatant was taken in another tube and kept for drying at 37 °C for 12 h. After drying 250 μl of assay buffer (10 mM PBS with 1% BSA) was added to re-constitute the steroid. Hepatic tissue from each fish was collected and stored in ice cold phosphate buffer until it was homogenized and sonicated at 4 °C in a homogenizing buffer (50 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 100 mM sucrose, 1 mM PMSF, and 1% leupeptin hemisulphate), for preparation of 10% tissue homogenate. The entire tissue sample was stored at −80 °C for further analysis of biochemical parameters.

### 2.5. Measurement of enzymatic and non-enzymatic antioxidants in the hepatic tissue

Supernatant of each of the liver tissue samples was used to quantify the levels of different enzymatic [superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd), glutathione peroxidase (GPx), glutathione S-transferase (GST)] and non-enzymatic antioxidants [malondialdehyde (MDA), glutathione (GSH)], according to standard methods described previously (Moniruzzaman et al., 2016).

#### 2.5.1. Malondialdehyde (MDA) level as the measure of lipid peroxidation

The supernatants of the follicular extracts were used to measure the level of MDA equivalents derived as a product of lipid peroxidation by TBARS (thiobarbituric acid reactive substances) assay with minor modifications as described earlier by Hasan et al. (2014).

#### 2.5.2. Antioxidative agents

The tissue samples were used separately for quantitative estimations of different antioxidative agents following well calibrated specific spectrophotometric methods. Each assay was validated by serial dilutions of the substrates and/or addition of selective inhibitors of respective enzymes.

#### 2.5.3. Enzymatic antioxidative agents

*2.5.3.1. Superoxide dismutase (SOD).* The SOD activity was measured following a spectrophotometric method based on assessment of $O_2.^-$-mediated nitro blue tetrazolium reduction by an aerobic mixture of NADH and PMS. The superoxide radicals were generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μM) solution, 1 ml NADH (78 μM) solution and sample extracts were mixed. The reaction started by adding 1 ml of PMS solution (10 μM) to the mixture and following incubation at 25 °C for 5 min the absorbance was measured at 560 nm against reagent blank. To validate the assay, serial dilutions of NADH and PMS were used (Hasan et al., 2014; Moniruzzaman et al., 2016).

#### 2.5.4. Catalase (CAT)

The catalase activity was measured using the method described elsewhere. Absorbance was monitored at 240 nm up to 90 s at 15 s intervals. For validation of assay, the tissue homogenates were treated with sodium azide, a known inhibitor of CAT activity (Hasan et al., 2014; Moniruzzaman et al., 2016).

#### 2.5.5. Glutathione peroxidase (GPx)

The GPx activity was measured following a method, in which absorbance was measured at 492 nm against blank (100 μl extra OPD solution instead of sample). Serial dilutions of the substrate (o-phenylenediamine; OPD) of GPx were used to validate the assay.

#### 2.5.6. Glutathione reductase (GRd)

The activity of GRd was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm, in a reaction mixture

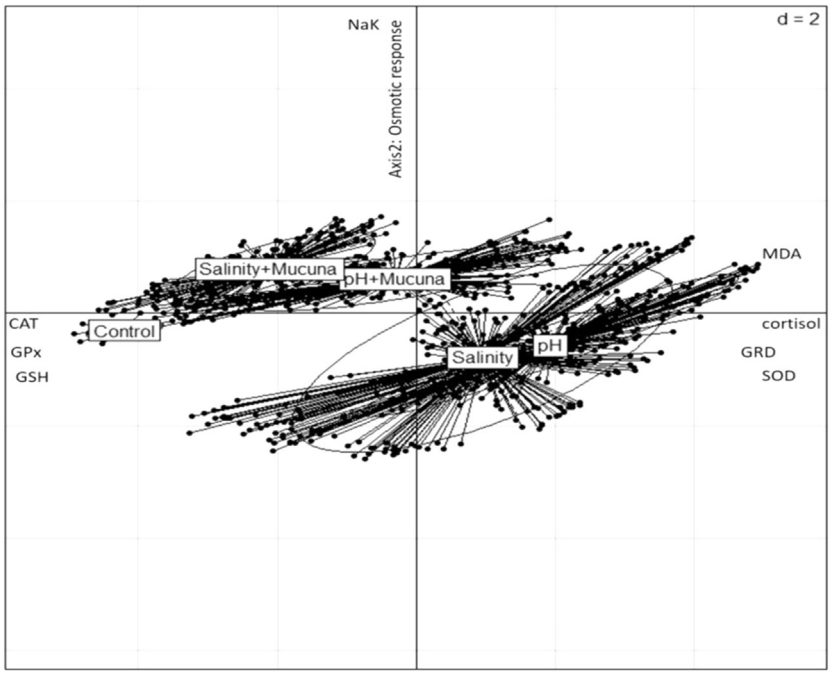


**Fig. 2.** Scatter plot representing the effects of treatments on the physiological responses of guppies submitted to a pH stress treatment or a saline stress treatment (all doses together) without *Mucuna* (pH, Salinity), or with *Mucuna* (pH + *Mucuna*, Salinity + *Mucuna*) or no stress treatment (Control) following a PCA analysis. Each individual is represented by a dot. Axis 1 represents mostly the intensity of the oxidative and hormonal stress response (SOD, CAT, GPx, MDA, GSH, GRD and Cortisol levels) which explains 63.64% of the total variance and axis 2 represents mostly the osmotic response (NaK: $Na^+$-$K^+$ATPase activity level) which explains 12.95% of the total variance. pH and Salinity groups are characterized by a higher stress response (right part of the panel), while *Mucuna* treated individuals are characterized by a lower stress response (left part of the panel) and higher osmotic response (upper part of the panel). Control individuals have the lowest stress response (extreme left of the panel).

containing 950 µl of 0.15 mM NADPH, 0.5 mM glutathione, and 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5) and 50 µl extract. Corrections were made for NADPH oxidation in the absence of glutathione.

#### 2.5.7. Non-enzymatic antioxidative agent (reduced glutathione; GSH)

Quantity of GSH, a faithful non-enzymatic antioxidative agent, in each follicular extract was measured following the method described earlier (Hasan et al., 2014). The level of GSH in each sample was calculated by extrapolating the data from the standard graph prepared using GSH.

### 2.6. Measurement of $Na^+$-$K^+$ATPase activity in the gill tissue

$Na^+$-$K^+$-ATPase activity, expressed as µmol Pi liberated/mg protein/h in the gill was measured by liberating $PO_4$ from a hydrolysis reaction with ATPase, as previously described (Agrahari and Gopal, 2008).

### 2.7. Immunoassay of 17-β estradiol ($E_2$), testosterone and cortisol (SH)

To measure reproduction-related traits, we focused on reproductive hormones and reproductive behaviours. Serum sample from each fish were collected and used for measurement of sex steroid [17 β-estradiol ($E_2$) and testosterone] using standardized Enzyme Immunoassay Kit (BiocheckInc, 837 Cowan Road, Burlingame, Ca 94010). The absorbance was measured at 450 nm and 495 nm respectively with Tecan-Spectra automatic Microplate reader. The intra- and inter assay coefficient of variations were 2.1% and 4.5% for 17β-estradiol and 1.8% and 3.3% for testosterone respectively.

The concentration of stress hormone cortisol was also determined in Microplate reader using Pars Azmoon Kits. The intra- and inter assay coefficient of variations of the assay were 1.9% and 5.1% respectively.

### 2.8. Reproductive behaviours

At the end of each experiment schedule (at day 7, 14, 21, 28, 35 and 42), fish (n = 9 females, 9 males) were isolated and kept in separate aquarium (30 × 30 × 30 cm) as individual pair to perform the mating trials. Mating trials with control fish (n = 9 females, 9 males) were performed at the beginning of the experiment (at day 0), after 21 days of stress experiment (at day 21), and after 21 days of recovery period (at day 42). Control fish showed no significant variations in mating behaviours at all these times. Mating behaviour of the male guppy is characterized mainly by gonopodial thrusts. As an important reproductive attributes, we observed the number of gonopodial thrusts performed by each male, which generally does not require the female reception. An attempt of mating was referred when the modified anal fin (gonopodium) of male made contact with the female genital region. The mating behaviour was observed after 24 h of the initial exposure. Each pair was observed in a randomly selected order. The number of gonopodial thrusts performed by each male in the tanks, were calculated over 15 min, using a counting device. Each male was observed two times a day (10.00 a.m., and 6.00 p.m.) and the average number of thrusts performed by each individual was calculated.

Three broods were produced at the end of the mating trials for each treatment categories. After the mating trials, females were isolated until giving birth. Gestation time was measured as the sum of the time from insemination to fertilization along with development time from fertilization to birth.

### 2.9. Statistical analyses

To understand the interaction of the physiological parameter variables (SOD, CAT, GPx, MDA, GSH, GRD, cortisol levels, and $Na^+$-$K^+$ATPase activity level) which are likely correlated, we started with a Principal component analysis (PCA), which is the most appropriate method for dimension reduction in space and indirect gradient analysis for integrated data interpretation.

Then we focused on specific variables reflecting different aspects of stress physiology to better understand the effects of treatments and duration of exposure to stressor on fish responses. We analysed cortisol levels (hormonal response), MDA levels (oxidative response), $Na^+$-$K^+$ATPase activity (osmoregulatory response) and gestation time or gonopodial thrusts (reproductive response) using Generalized Linear Models GLM. Sex, stressor (pH or salinity), dose of stress (low, moderate or high), time (7, 14 or 21 days), *Mucuna* treatment (with or without *Mucuna*) and *Mucuna** dose and *Mucuna**time interactions were added as explanatory variables. Non significant terms (P values

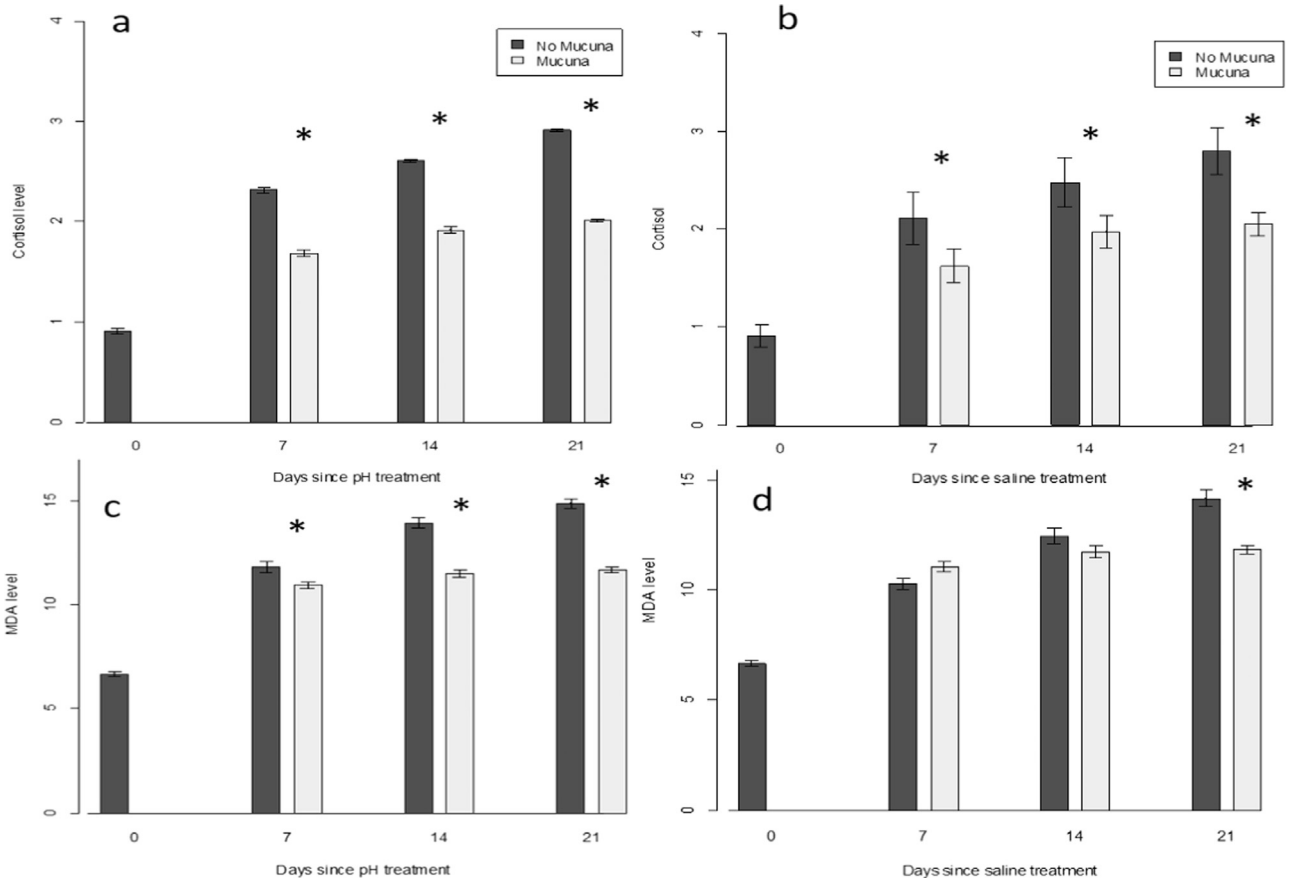


**Fig. 3.** Cortisol (a and b) or MDA levels (c and d) in response to (a and c) pH and (b and d) saline stress (all doses together) applied for different durations (0: control, 7 days, 14 days or 21 days) without *Mucuna* (dark grey) or with *Mucuna* treatment (light grey bars). Asterisks represent significant differences ($P < 0.05$) between *Mucuna* groups.

$> 0.05$) were removed and only final models are presented in the Tables. Mean ± SD values for all the variables were presented in the graphs. Means were compared by a *post hoc* multiple range test taking $p < 0.05$ as the threshold. All the analyses have been done using R (R Development Core Team 2013) (Fig. 2).

## 3. Result

### 3.1. Multivariate analysis on physiological markers

The enzymatic and non-enzymatic antioxidants (SOD, CAT, GPx, GRD, GSH, GST and MDA levels) in the hepatic tissue changed significantly ($P < 0.05$) after exposure to both pH and salinity stress. *Mucuna* treatment restored those levels near to control values (Supplementary material, Fig. S1 and Fig. 3c –d)

The first axis of the PCA represented mostly the intensity of the oxidative and hormonal stress response (SOD, CAT, GPx, MDA, GSH, GRD and cortisol levels) and explained 63.64% of the total variance while second axis represented mostly the osmotic response ($NA^+$-$K^+$ATPase activity level) and explained 12.95% of the total variance (Fig. 2). Control individuals had the lowest oxidative stress response (Fig. 2, extreme left of the panel). pH and salinity-exposed groups exhibited a higher stress response (Fig. 2, right part of the panel) compared to the control group, while *Mucuna* treated individuals were characterized by a lower stress (Fig. 2, left part of the panel) and higher osmotic response (Fig. 2, upper part of the panel) compared to the pH and salinity treated groups.

### 3.2. Cortisol levels

pH stress (Generalized Linear Model, effect of pH stress: $t = 9.98$, $P < 0.001$) and salinity stress (GLM: $t = 9.49$, $P < 0.001$) increased guppies cortisol levels compared to controls, and pH induced a stronger cortisol response compared to salinity stress ($t = 7.34$, $P < 0.001$). For both stressors, cortisol levels increased with stress duration, and males had higher serum cortisol levels than females (Table 1).

*Mucuna* treatment significantly reduced cortisol levels in pH-stressed and in salinity-stressed fish (Table 1, Fig. 3a). This effect was significant at all pH and all salinity doses (all $P < 0.001$) and for all stress durations (all $P < 0.001$) (Fig. 3a and b).

### 3.3. Oxidative stress response and MDA oxidative damages

pH and salinity stressors increased guppies MDA levels reflecting oxidative damages compared to controls, and pH induced a stronger increase in MDA level compared to salinity stressor (GLM: estimate ± SE = 4.11 ± 0.41, $t = 9.88$, $P < 0.001$). MDA levels increased in all stress-exposed fish and the effects were dose and duration-dependent (Table 1).

*Mucuna* treatment significantly reduced MDA levels and this effect was stronger at the highest dose of pH stress (GLM: High dose: − 4.22 ± 0.22, $t = -19.48$, $P < 0.001$), but was effective at all pH stress doses (all $P < 0.001$). *Mucuna* treatment had effects only for a high dose of salinity (Low Dose: estimate ± SE = − 0.20 ± 0.24, $t = -0.86$, $P = 0.39$; Medium Dose: estimate ± SE = 0.29 ± 0.34, $t = 0.84$, $P = 0.41$; High dose: estimate ± SE = − 2.38 ± 0.29, $t = -8.31$, $P < 0.001$).

*Mucuna* treatment caused a significant reduction of MDA levels for

**Table 1**
Best Generalized Linear Models model explaining Cortisol, MDA and $Na^+$-$K^+$ ATPase activity levels of guppies exposed to an acid (pH) or osmotic (salinity) stress at different doses (low, medium or high) and for different durations (0, 7, 14 or 21 days) with or without *Mucuna*.

| Response variable | Predictors | Acid stress (pH treatment) | | | Osmotic stress (salinity treatment) | | |
|---|---|---|---|---|---|---|---|
| | | Estimate ± SE | t value | p value | Estimate ± SE | t value | p value |
| Cortisol | Sex (M) | 0.32 ± 0.038 | 8.50 | < 0.001 | 0.21 ± 0.034 | 6.12 | < 0.001 |
| | *Mucuna* | − 0.33 ± 0.13 | − 2.53 | 0.011 | − 0.51 ± 0.12 | − 4.26 | < 0.001 |
| | Dose of stressor | 0.62 ± 0.026 | 23.61 | < 0.001 | 0.63 ± 0.024 | 26.13 | < 0.001 |
| | Stress duration | 0.041 ± 0.004 | 8.62 | < 0.001 | 0.047 ± 0.0043 | 11.04 | < 0.001 |
| | *Mucuna** Dose | − 0.39 ± 0.043 | − 9.12 | < 0.001 | − 0.41 ± 0.039 | − 10.47 | < 0.001 |
| | *Mucuna**Duration | − 0.018 ± 0.007 | − 2.61 | 0.0092 | − 0.018 ± 0.006 | − 2.87 | 0.0043 |
| MDA | Sex (M) | 0.78 ± 0.13 | 5.66 | < 0.001 | 0.39 ± 0.13 | 2.97 | 0.003 |
| | *Mucuna* | 1.47 ± 0.48 | 3.046 | 0.002 | 4.58 ± 0.47 | 9.81 | < 0.001 |
| | Dose of stressor | 3.17 ± 0.097 | 32.73 | < 0.001 | 2.90 ± 0.094 | 30.96 | < 0.001 |
| | Duration | 0.15 ± 0.017 | 8.69 | < 0.001 | 0.28 ± 0.017 | 16.39 | < 0.001 |
| | *Mucuna** Dose | − 1.71 ± 0.16 | − 10.93 | < 0.001 | − 1.12 ± 0.15 | − 7.40 | < 0.001 |
| | *Mucuna**Duration | − 0.10 ± 0.025 | − 4.16 | < 0.001 | − 0.22 ± 0.024 | − 9.25 | < 0.001 |
| $Na^+$-$K^+$ ATPase activity | *Mucuna* | 0.86 ± 0.034 | 25.12 | < 0.001 | 0.83 ± 0.051 | 16.16 | < 0.001 |
| | Dose of stressor | 0.07 ± 0.006 | 10.19 | < 0.001 | 0.080 ± 0.010 | 7.66 | < 0.001 |
| | Duration | 0.034 ± 0.0016 | 21.21 | < 0.001 | 0.028 ± 0.0024 | 11.85 | < 0.001 |
| | *Mucuna**Duration | − 0.025 ± 0.002 | − 11.22 | < 0.001 | − 0.019 ± 0.0034 | − 5.64 | < 0.001 |

all stress durations in pH stressed fish (all $P < 0.001$) (Fig. 3c), but was only significant for a 21 days duration of exposure to salinity stress (GLM: estimate ± SE = − 2.33 ± 0.42, t = − 5.51, $P < 0.001$) (Fig. 3d).

### 3.4. Osmotic stress response: $Na^+$-$K^+$ATPase activity

$Na^+$-$K^+$ATPase activity increased with the duration of stress and pH and salinity effects were dose dependent (Table 1). *Mucuna* treatment increased $Na^+$-$K^+$ATPase activity in pH- and salinity-stressed fish at all the experimental doses and at stress durations (Fig. 4a and b), but this effect was stronger for a stress duration of 7 days (Day 7: estimate ± SE = 0.72 ± 0.022, t = 32.42, $P < 0.001$; Day 14: estimate ± SE = 0.58 ± 0.021, t = 27.71, $P < 0.001$; Day 21: estimate = 0.43 ± 0.019, t = 22.71, $P < 0.001$).

### 3.5. Reproduction

Gestation time in females increased while gonopodial thrusts decreased with the dose and duration of pH and salinity stress (Table 2). *Mucuna* treatment decreased gestation time and increased the number of gonopodial thrusts at all treatment durations and doses but the effect of *Mucuna* increased over the time (Table 2; posthoc tests: all $P < 0.001$) (Fig. 5).

Analyses on reproductive hormones bring similar results (Supplementary material, Fig. S2). 17β-estradiol level decreased significantly ($P < 0.05$) in a duration and dose dependent manner of both the pH and salinity treatment (Supplementary material, Fig. S2). Similar drop ($P > 0.05$) in testosterone level was also observed after the respective treatment schedule (Supplementary material, Fig. S2). *Mucuna* extract significantly reinstated the levels of the 17β-estradiol and testosterone though a significant ($P < 0.05$) effect was observed at least after 14 days of treatment. However, the effect was stronger after 21 days of treatment (Supplementary material, Fig. S2).

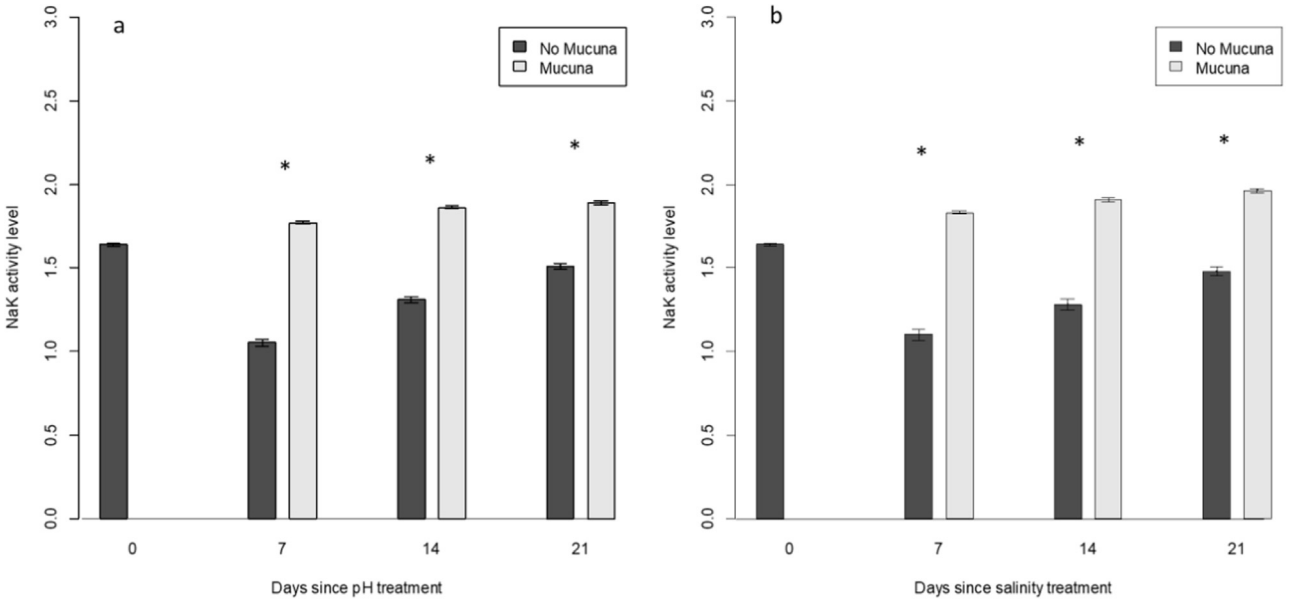


**Fig. 4.** $Na^+$-$K^+$ATPase activity levels across time in response to (a) pH and (b) saline stressors treatments (all doses together) applied for different durations (0: control, 7 days 14 days or 21 days) without *Mucuna* (dark grey) or with *Mucuna* treatment (light grey bars). Asterisks represent significant differences ($P < 0.05$) between *Mucuna* groups.

**Table 2**
Best Generalized Linear Models explaining gestation time in females and gonopodial thrusts in male guppies exposed to an acid (pH) or osmotic (salinity) stress.

| | Acid stress (pH treatment) | | | Osmotic stress (salinity treatment) | | |
|---|---|---|---|---|---|---|
| Predictors | Estimate ± SE | t value | p value | Estimate ± SE | t value | p value |
| *Females* | | | | | | |
| *Mucuna* | 2.17 ± 0.72 | 3.02 | 0.0028 | − 0.13 ± 0.71 | − 0.18 | 0.85 |
| Dose of stressor | 0.36 ± 0.17 | 2.16 | 0.032 | 1.35 ± 0.16 | 8.12 | < 0.001 |
| Duration | 0.33 ± 0.031 | 10.97 | < 0.001 | 0.26 ± 0.030 | 8.58 | < 0.001 |
| *Mucuna**Duration | − 0.35 ± 0.048 | − 7.23 | < 0.001 | − 0.19 ± 0.047 | − 5.64 | < 0.001 |
| *Males* | | | | | | |
| *Mucuna* | 0.11 ± 0.035 | 3.03 | 0.0027 | 0.037 ± 0.051 | 0.71 | 0.47 |
| Dose of stressor | − 0.064 ± 0.008 | − 7.74 | < 0.001 | − 0.046 ± 0.012 | − 3.87 | < 0.001 |
| Duration | − 0.013 ± 0.001 | − 9.035 | < 0.001 | − 0.014 ± 0.0022 | − 6.52 | < 0.001 |
| *Mucuna**Duration | 0.022 ± 0.002 | 9.46 | < 0.001 | 0.025 ± 0.0034 | 7.33 | < 0.001 |

## 4. Discussion

We identified key physiological traits (cortisol, MDA, $Na^+$-$K^+$ATPase, reproductive behaviours and hormones) responding to environmental pH and salinity changes in guppies. Such eco-physiological parameters might thus be used as indicators of environmental stress in the wild or in captivity and be used as biomarkers for future monitoring in aquatic systems. Second, we showed that *Mucuna* plant extract decreased the levels of these biomarkers of stress in guppies. Such treatment might thus potentially be used in captive fish to improve their physiological condition when exposed to increased salinity or acid stress in the context of global change.

### 4.1. Sex-specific cortisol response

Generally, most of the aquatic animals including fish respond to acute and chronic stressors with a rapid elevation of blood cortisol levels (Calcagno et al., 2016). Basal levels of cortisol depend on a combination of abiotic factors of the aquatic system (Tellis et al., 2012). In the current study, cortisol levels increased in guppy exposed to pH and salinity stress. Interestingly, plasma cortisol concentration was higher in males than in females which is consistent with previous studies (Chouinard-Thuly et al., 2018). Earlier investigations have also indicated a strong negative correlation between cortisol and male sex steroid (Mandal et al., 2017; Baduy et al., 2017). The high levels of cortisol in males suggested that the mechanism for synthesis of cortisol might be strongly influenced through hypothalamus-pituitary-interrenal (HPI) axis, especially at the onset of reproductive-active period which might be controlled by certain environmental factors like pH and salinity.

Earlier studies (Beaulieu-McCoy et al., 2017) have reported variations in cortisol response to different stressors in mature males, which indicated that the inhibitory effect of stress on reproductive physiology were interceded by cortisol. Such effect did not involve inhibition of GtH secretion, but possibly acted at the level of GtH signal-transduction in gonadal tissue and altered secretion of sex steroids. During breeding season, some fish can respond to stress with elevated cortisol levels and decreased sex steroid levels. Unlike catecholamines and other

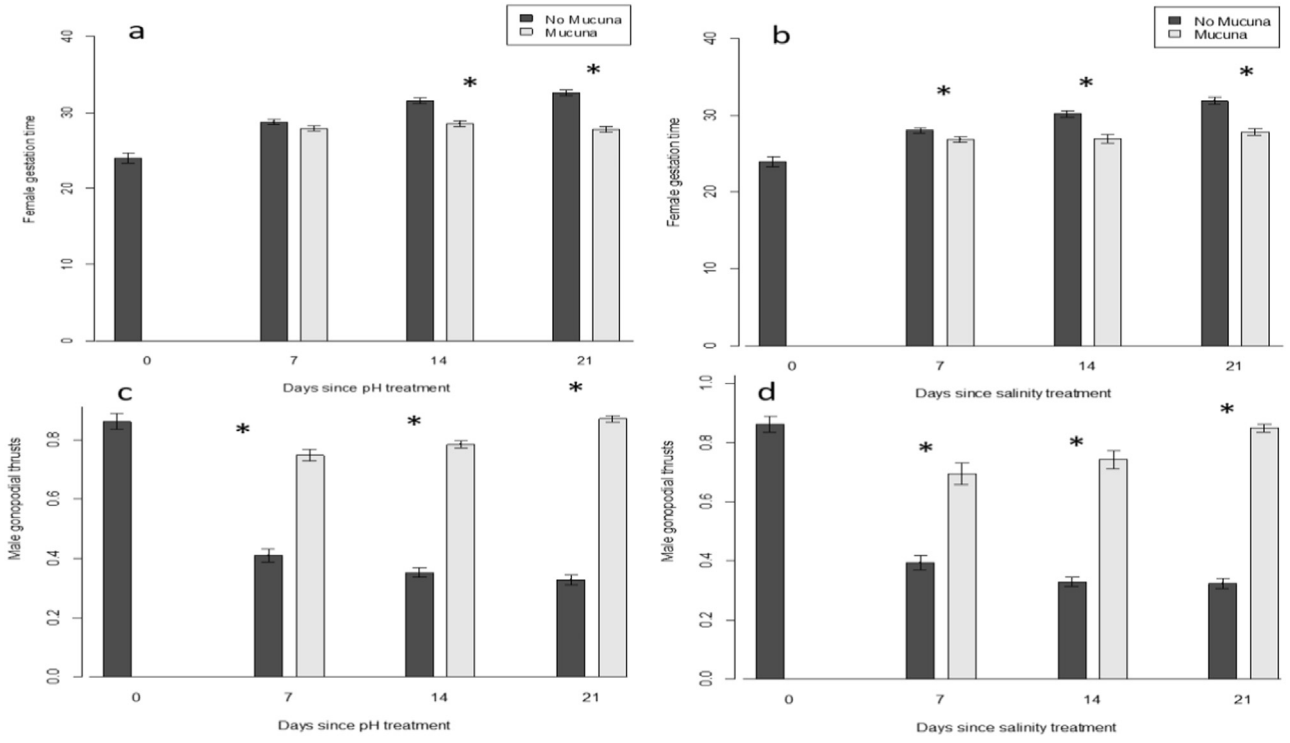


**Fig. 5.** Gestation time of females (a and b) and gonopodial thrust of males (c and d) across time in response to pH (a and c) and salinity (b and d) stressors (all doses together) applied for different durations (0: control, 7 days 14 days or 21 days) without *Mucuna* (dark grey) or with *Mucuna* treatment (light grey bars). Asterisks represent significant differences ($P < 0.05$) between *Mucuna* groups.

corticosteroids, cortisol is released gradually and remains elevated for extended period throughout the stress (Lataretu et al., 2013). Therefore, cortisol is commonly considered as the mediator between stress and other physiological activities in vertebrates particularly in fishes (Kijewska et al., 2016). Here our results suggest that the release of cortisol triggers secondary responses at oxidative status in the hepatic tissue, such as alterations to free radical and hydroxyl ions and the expression of different stress proteins. This could participate in the acclimation process to environmental stressors. Secondary responses are energetically costly and if the stressor persists the increased energy expenditure may lead to tertiary responses which can have consequences on the respiration process in the gill tissue. This in turn affects the reproductive physiology and behaviour of the organisms through the modulation of endocrine status.

### 4.2. Oxidative stress response

Liver plays crucial role to regulate homeostasis process and maintain natural body physiology. Hepatic tissue is metabolically the most active organ and is responsible for the breakdown of metabolites and toxic elements. Thereby liver has the highest ROS production rate in the body and is the most preferred organ to assess the status of oxidative stress and antioxidant defense mechanism in aquatic organisms. Increased lipid peroxidation is a recurrent and quick response to ROS generation (Moniruzzaman et al., 2017). Ions of different transition substrates are responsible for excess lipid peroxidation due to the formation and overload of hydroxyl radical via the Fenton reaction (Kehrer, 2000). It is thus highly relevant for biomonitoring studies on environmental stress (Sinha et al., 2015). In the current study MDA levels in the liver of guppies exposed to pH and salinity stress increased both with dose and duration of the stress. This could be the result of hyper osmotic and hyper acidic environment and ultimately promulgates a pro-oxidant condition. Accordingly, MDA level was reported to increase in hepatic tissue of gilthead seabream (*Sparus aurata*) during nutritional stress (Guardiola et al., 2017). Salinity stress was also reported to considerably enhance the rate of lipid peroxidation in sturgeons (*Acipenser naccarii*) (Martinez-Alvarez et al., 2002). In this study, we further showed that salinity and acid stress could also have such effects on oxidative stress, a result that had not been studied comprehensively in previous studies. In addition, MDA levels were higher in males compared to females that are consistent with their higher cortisol levels. Previous studies reported sex-specific values of oxidative stress, specifically MDA during male maturational process (Christensen et al., 2016). The present study confirms this hypothesis of a higher sensitivity of male to oxidative stress, possibly due to the anti-oxidant effects of sex steroid 17β-estradiol (Pronsato et al., 2016).

### 4.3. Osmoregulation

$Na^+$-$K^+$ATPase activity represents the osmoregulatory response (Rind et al., 2017). We observed a decreased activity of $Na^+$-$K^+$ATPase after both acidic and hyper salinity stress which could be due to an enhancement of oxidative load. Moreover, impairment of oxidative status and $Na^+$-$K^+$ATPase activity after pH and salinity stress might be associated with an alteration in oxygen consumption rate in the gill tissue. Further experimental data on oxygen consumption rate are needed to test this hypothesis. Gills are active respiratory and osmoregulatory organ that are prone to oxidative injuries particularly during acidic or osmotic stress (Paital and Chainy, 2012). $Na^+$-$K^+$ATPase generally maintains ion permeability in the cellular membrane, relative stability of various ion concentrations in the intracellular environment and osmotic pressure balance between intracellular and external environments (Geng et al., 2016). Therefore, altered $Na^+$-$K^+$ATPase activity might be due to the sudden adjustment needed in the chloride cells of the gill filament when ion concentrations in the external environment suddenly fluctuate. Cortisol also plays a key role in gill osmoregulatory mechanism and normally stimulates gill $Na^+$-$K^+$ATPase activity (Pankhurst, 2011). Acidic or hyper osmotic stress applied in this study could thus have altered the level of cortisol and cause a subsequent reduction of $Na^+$-$K^+$ATPase activity. Previous study already reported biochemical and physiological fluctuations in gills of euryhaline species in response to salinity stress in order to maintain osmotic and ionic homeostasis and causing an alteration in acid-base balance (Paital and Chainy, 2012).

### 4.4. Reproductive behaviours and hormones

Key reproductive attributes (gestation time in females and gonopodial thrusts in males) were strongly affected by the pH and salinity stress. Fish exposed to pollutants generally display a range of such reproductive dysfunction (Carnevali et al., 2018; Flint et al., 2018; Kellock et al., 2018). Our results clearly indicate that environmental stress like pH or salinity also induce a dysfunction in reproductive behaviour that could have strong consequences for reproductive potential and fitness in the wild and in captivity (Salleh et al., 2017). To understand the underlying mechanism behind such alterations we measured sex steroid hormones in both male and female guppy. Low levels of sex steroid hormones were detected in fish of both sexes under osmotic and acidic stress. Such reduction of sex steroid synthesis might be the cause of impairment of reproductive behaviours in guppy. Our study confirmed the previous hypothesis regarding the interplay between hormonal and behavioural profiles observed in natural populations under variable environmental conditions. Therefore, the observed correlations between behavioural profiles in different sexes, respective sex steroid profile and stress hormone responses within guppy populations are the central regulators of such reproductive behaviour alteration under pH or salinity stress.

### 4.5. Relevant biomarkers of acid versus salinity stress

Guppy generally prefers a pH in a range between 6.8 and 7.5. Guppies can tolerate a pH around 6.0 but a lower pH is likely very stressful for this species (Froese et al., 2017). The tolerable limit of salinity for guppy is around 1–1.5 ppt. Guppy can survive up to a salinity range of 4.5–5.0 ppt, though it might be stressful and hamper the normal physiological activity of the fish (De Silva and Samayawardhena, 2005). Changes in different physiological parameters at each biological level suggest that pH changes induce higher stress responses in guppies compared to salinity changes. Similar results were also found in some other species as well; where aquatic species exposed to variable pH showed greater decrease in different immunological factors and overall health status compared to species exposed to variable osmotic stress (Sharma and Sohn, 2009). Moreover, stress caused by altered pH may intensely inhibit antioxidant markers because acidic water also impairs oxygen entry, oxygen transfer and oxygen carrying capacity (Sharma and Sohn, 2009). Therefore, this study helps specifying the appropriate pH or salinity levels in the aquatic environment that are within the tolerance range of this species to maintain the body physiology. It also shows that cortisol, MDA, $Na^+$-$K^+$ATPase and reproductive behaviour is relevant biomarkers of both acidic and osmotic stress in the environment. Such biomarkers could thus be used to predict and prevent subsequent alterations in fish health and reproductive potency that could be detrimental for populations both in the wild and in captivity.

### 4.6. Mucuna effects in reducing stress responses

Our results show that *Mucuna* extract have strong and positive effects on all stress biomarkers studied. For instance, *Mucuna* treatment decreased cortisol levels, MDA levels, increased $Na^+$-$K^+$ATPase activity and reproductive markers. These results are consistent with our previous studies in carp and catfish that have demonstrated that

administration of *Mucuna* seed extract to SDS-treated fish resulted in a significant decrease in MDA levels, GST and GRd activities; and an increase in activities of SOD, CAT and other neurological parameters regulating the brain function (Kumar et al., 2016; Mukherjee et al., 2017a, 2017b). These observations also indicated that with its antioxidant property, the plant extract could play a crucial role in restoring the physiological activity and protect the tissue from possible damage associated with acid and salinity stress. The antioxidant and neuroprotective activity of the extracts may be attributed to the presence of specific phytochemicals such as flavonoids, terpenoids and tannins in *Mucuna* seed extracts (Dai and Mumper, 2010). Phytochemicals might contribute to maintain the cellular antioxidative status and osmoregulatory balance in guppy. They could stabilize these parameters after acute acidic and salinity stress and help to maintain the architecture of the cellular components (Kunjiappan et al., 2015). Possibly both these neuro-protective and antioxidant activities of *Mucuna* are the major controlling factors in restoring the levels of both male and female sex steroids nearer to the basal level, which were altered after pH and salinity stress, although further studies are now needed to test this hypothesis.

Phytochemical extracts are likely to protect the cell membranes against the production of free radicals; but could also regulate the activation of the transcription factors associated with expression of several antioxidant enzymes (Forman et al., 2014). Molecular mechanisms involved in oxidative damage, free radical accumulation and the associated transduction pathways, which regulate intermediary metabolism during stress related anoxia/hypoxia, might also be involved in the activation and maintenance of antioxidant enzymes and GSH levels in response to pH and salinity stress. Although shifts in protein expression at different functional levels are a common sign of adaptive processes, they are not the only processes involved in stress tolerance. Phytochemical possibly alters different transcription factors such as MAPK, AP-1, NRF2, NF-kappa-B and HIF-1 to modulate the stress response (Pratheeshkumar et al., 2014; Kunnumakkara et al., 2017). Activation and/or maintenance of antioxidants are often involved in tolerance and survival to oxidative stress and are key to the maintenance of the associated physiological imbalance. Another possible mechanism is through the modulation in steroid receptor binding efficiency of some of the components of the selected phytochemicals. Overall, our results could indicate delicate molecular changes in steroid receptor structure that can have substantial effects on the receptor-ligand binding affinity as well as function. Further research will help to elucidate the proper molecular mechanisms involved in the regulation of oxyradical detoxification metabolism by phytochemicals.

A duration of 7 days seemed to be effective for *Mucuna* to restore the physiological attributes and it might be due to the stretch of time required for phytochemicals to stimulate different transcription factors regulating antioxidants and other signalling proteins (Katiyar, 2016). In addition, *Mucuna*-enriched feed was more effective against salinity stress, although the underlying mechanism remains to be tested in future. Aquatic habitat will increasingly be exposed to salinity and pH alterations in the near future due to increased anthropogenic activities or climatic shift. Alternative feeding treatment with such plant extract could provide help maintain physiological and reproductive status of different wild as well as cultured fish.

## 5. Conclusion

This study helped quantifying the physiological responses to environmental acidic and osmotic stress in an aquatic species by a combination of different biomarkers. Such quantification could be useful to indicate levels of water alterations in wild and captive conditions and to anticipate their physiological consequences on aquatic organisms. Here, cortisol, lipid peroxidation (MDA levels), $Na^+$-$K^+$ATPase and reproductive behaviours showed consistent patterns of response to acidic and osmotic stressors and seem to be effective biomarkers of environmental changes. It suggests that the physiological or reproductive dysfunctions caused by these stressors might be due to the loss of oxidative balance and an overproduction of ROS. In addition, results show that alternative feeding with *Mucuna* plant extract could help maintaining the proper physiological balance of freshwater fish under fluctuating environmental conditions. Such feed modification could be a key factor limiting reproductive impairment and oxidative load caused by environmental hyper-osmotic and acid stress that are expected to increase in the near future due to global change.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## Funding

MM thankfully acknowledges DBT Research Associateship Programme, Govt. of India, IISC, Bangalore, for financial support. LJ was supported by a Toulouse University Idex starting grant (IDEX-V5RJACQ). This work was supported by the French National Program CNRS EC2CO-ECODYN and by a AEAG grant (PHYPAT-RECAC16P0068 and RECAC17P0154). The EDB laboratory is supported by the LABEX TULIP (ANR-10-LABX-41). JM is thankful to Visva-Bharati University and Postdoctoral Fellowship for Women (bearing award letter no. F. 151/2014-15/PDFWM-2014-15-GE-WES-25053(SA-II)) funded by University Grant Commission, New Delhi, India for financial support to carry out the work.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.07.053.

## References

Agrahari, S., Gopal, K., 2008. Inhibition of $Na^+$-$K^+$ATPase in different tissues of freshwater fish *Channa punctatus* (Bloch) exposed to monocrotophos. Pest. Biochem. Physiol. 92, 57–60.

Allen, J.D., Schrage, K.R., Foo, S.A., Watson, S.A., Byrne, M., 2017. The effects of salinity and ph on fertilization, early development, and hatching in the crown-of-thorns seastar. Diversity 9, 13.

Babu, P.V., Sabitha, K.E., Shyamaladevi, C.S., 2006. Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca 2+-ATPase and Na+/K +-ATPase activity in the heart of streptozotocin-diabetic rats. Chem. Biol. Interact. 162, 157–164.

Baduy, F., Guerreiro, P.M., Canário, A.V., Saraiva, J.L., 2017. Social organization and endocrine profiles of *Australoheros facetus*, an exotic freshwater fish in southern Portugal. Acta Ethologica. 20, 263–277.

Beaulieu-McCoy, N.E., Sherman, K.K., Trego, M.L., Crocker, D.E., Kellar, N.M., 2017. Initial validation of blubber cortisol and progesterone as indicators of stress response and maturity in an otariid; the California sea lion (*Zalophus californianus*). Gen. Comp. Endocrinol. 252, 1–11.

Birben, E., Umit Murat, S., Cansin, S., Serpil, E., Omer, K., 2012. Oxidative stress and antioxidant defense. WAO J. 5, 9–19.

Calcagno, E., Durando, P., Valdés, M.E., Franchioni, L., de los Ángeles Bistoni, M., 2016. Effects of carbamazepine on cortisol levels and behavioral responses to stress in the fish *Jenynsia multidentata*. Physiol. Behav. 158, 68–75.

Carnevali, O., Santangeli, S., Forner-Piquer, I., Basili, D., Maradonna, F., 2018. Endocrine-disrupting chemicals in aquatic environment: what are the risks for fish gametes? Fish. Physiol. Biochem. 1–16.

Chakraborty, S.B., Hancz, C., 2011. Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in fin fish culture. Rev. Aquacult. 3, 103–119.

Chakraborty, S.B., Horn, P., Hancz, C., 2014. Application of phytochemicals as growth-promoters and endocrine modulators in fish culture. Rev. Aquacult. 6, 1–19.

Chouinard-Thuly, L., Reddon, A.R., Leris, I., Earley, R.L., Reader, S.M., 2018. Developmental plasticity of the stress response in female but not in male guppies. R. Soc. Open Sci. 5, 172268.

Christensen, L.L., Selman, C., Blount, J.D., Pilkington, J.G., Watt, K.A., Pemberton, J.M., Nussey, D.H., 2016. Marker-dependent association among oxidative stress, growth and survival during early life in a wild mammal. Proc. R. Soc. B 283, 20161407.

Dadras, H., Dzyuba, B., Cosson, J., Golpour, A., Siddique, M.A.M., Linhart, O., 2017. Effect of water temperature on the physiology of fish spermatozoon function: a brief review. Aquac. Res. 48, 729–740.

Dai, J., Mumper, R., 2010. Plant phenolics: extraction, analysis and their antioxidant and

anticancer properties. Molecules 15, 7313–7352.
De Silva, P.M., Samayawardhena, L.A., 2005. Effects of chlorpyrifos on reproductive performances of guppy (*Poecilia reticulata*). Chemosphere 58, 1293–1299.
Feely, R.A., Alin, S.R., Newton, J., Sabine, C.L., Warner, M., Devol, A., Krembs, C., Maloy, C., 2010. The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. Estuar. Coast. Shelf Sci. 88, 442–449.
Flint, N., Pearson, R.G., Crossland, M.R., 2018. Reproduction and embryo viability of a range-limited tropical freshwater fish exposed to fluctuating hypoxia. Mar. Freshw. Res. 69, 267–276.
Forman, H.J., Davies, K.J., Ursini, F., 2014. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging *in vivo*. Free Radic. Biol. Med. 66, 24–35.
Froese, R., Demirel, N., Coro, G., Kleisner, K.M., Winker, H., 2017. Estimating fisheries reference points from catch and resilience. Fish Fish. 18, 506–526.
Geng, C., Tian, Y., Shang, Y., Wang, L., Jiang, Y., Chang, Y., 2016. Effect of acute salinity stress on ion homeostasis, Na+/K+-ATPase and histological structure in sea cucumber *Apostichopus japonicus*. SpringerPlus 5, 1977.
Guardiola, F.A., Bahi, A., Messina, C.M., Mahdhi, A., Santulli, A., Arena, R., Bakhrouf, A., Esteban, M.A., 2017. Quality and antioxidant response of gilthead seabream (*Sparus aurata* L.) to dietary supplements of fenugreek (*Trigonella foenum graecum*) alone or combined with probiotic strains. Fish. Shellfish Immunol. 63, 277–284.
Guevara-Fiore, P., Endler, J.A., 2018. Female receptivity affects subsequent mating effort and mate choice in male guppies. Anim. Behav. 140, 73–79.
Guinotte, J.M., Fabry, V.J., 2008. Ocean acidification and its potential effects on marine ecosystems. Ann. NY Acad. Sci. 1134, 320–342.
Halliwell, B., Gutteridge, J.M.C., 2006. Free Radicals in Biology and Medicine, 4th ed. Clarendon, Oxford.
Hasan, K.N., Moniruzzaman, M., Maitra, S.K., 2014. Melatonin concentrations in relation to oxidative status and oocyte dynamics in the ovary during different reproductive phases of an annual cycle in carp *Catla catla*. Theriogenology 82, 1173–1185.
Katiyar, S.K., 2016. Emerging phytochemicals for the prevention and treatment of head and neck cancer. Molecules 21, 1610.
Katoh, F., Cozzi, R.R.F., Marshall, W.S., Goss, G.G., 2008. Distinct $Na^+/K^+/2Cl^-$cotransporter localization in kidneys and gills of two euryhaline species, rainbow trout and killifish. Cell Tissue Res. 334, 265–281.
Kavitha, P., Subramanian, P., 2011. Influence of *Tribulus terrestris* on testicular enzyme in fresh water ornamental fish *Poecilia latipinna*. Fish. Physiol. Biochem. 37, 801–807.
Kehrer, J.P., 2000. The Haber–Weiss reaction and mechanisms of toxicity. Toxicology 149, 43–50.
Kellock, K.A., Moore, A.P., Bringolf, R.B., 2018. Chronic nitrate exposure alters reproductive physiology in fathead minnows. Environ. Pollut. 232, 322–328.
Kelly, V.R., Lovett, G.M., Weathers, K.C., Findlay, S.E., Strayer, D.L., Burns, D.J., Likens, G.E., 2007. Long-term sodium chloride retention in a rural watershed: legacy effects of road salt on stream water concentration. Environ. Sci. Technol. 42, 410–415.
Kijewska, A., Kalamarz-Kubiak, H., Arciszewski, B., Guellard, T., Petereit, C., Wenne, R., 2016. Adaptation to salinity in Atlantic cod from different regions of the Baltic Sea. J. Exp. Mar. Bio Ecol. 478, 62–67.
Klein, R.D., Rosa, C.E., Colares, E.P., Robaldo, R.B., Martinez, P.E., Bianchini, A., 2017. Antioxidant defense system and oxidative status in Antarctic fishes: the sluggish rockcod *Notothenia coriiceps* versus the active marbled notothen *Notothenia rossii*. J. Therm. Biol. 68, 119–127.
Kumar, S., Moniruzzaman, M., Mukherjee, M., Das, D., Chakraborty, S.B., 2016. *Mucuna* seed extract treatment alleviates SDS-induced oxidative stress and neuronal damage in carp brain. IJPPR 8, 1669–1674.
Kunjiappan, S., Bhattacharjee, C., Chowdhury, R., 2015. Hepatoprotective and antioxidant effects of *Azolla microphylla* based gold nanoparticles against acetaminophen induced toxicity in a fresh water common carp fish (*Cyprinus carpio L.*). Nanomedicine 2, 88–110.
Kunnumakkara, A.B., Bordoloi, D., Harsha, C., Banik, K., Gupta, S.C., Aggarwal, B.B., 2017. Curcumin mediates anticancer effects by modulating multiple cell signaling pathways. Clin. Sci. 131, 1781–1799.
Kwong, R.W.M., Kumai, Y., Perry, S.F., 2014. The physiology of fish at low pH: the zebrafish as a model system. J. Exp. Biol. 217, 651–662.
Lataretu, A., Furnaris, F., Mitranescu, E., 2013. Hematologic profile as stress indicator in fish. Scientific works. Ser. C Vet. Med. 59, 102–104.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.
Madhyastha, S., Chauhan, R., Rao, G.M., Umesh, H., 2011. Neuroprotective effects of *Mucuna pruriens* against stress-induced oxidative damage. J. Physiol. Biomed. Sci. 24, 28–33.
Makrinos, D.L., Bowden, T.J., 2016. Natural environmental impacts on teleost immune function. Fish. Shellfish Immunol. 53, 50–57.
Mandal, B., Sawant, P.B., Dasgupta, S., Chadha, N.K., Sundaray, J.K., Sawant, B.T., Bera, A., 2017. Deviation of habitat salinity during seasonal gonad recrudescence affects plasma sex steroid levels and suppresses gonadal maturation in an euryhaline fish *Etroplus suratensis*. Aquac. Res. 48, 5973–5983.
Martinez-Alvarez, R.M., Hidalgo, M.C., Domezain, A., Morales, A.E., García-Gallego, M., Sanz, A., 2002. Physiological changes of sturgeon *Acipenser naccarii* caused by increasing environmental salinity. J. Exp. Biol. 205, 3699–3706.
Miller, G.M., Kroon, F.J., Metcalfe, S., Munday, P.L., 2015. Temperature is the evil twin: effects of increased temperature and ocean acidification on reproduction in a reef fish. Ecol. Appl. 25, 603–620.
Moniruzzaman, M., Ghosal, I., Das, D., Chakraborty, S.B., 2018. Melatonin ameliorates $H_2O_2$-induced oxidative stress through modulation of Erk/Akt/NFkB pathway. Biol. Res. 51, 17.
Moniruzzaman, M., Hasan, K.N., Maitra, S.K., 2016. Melatonin actions on ovaprim (synthetic GnRH and domperidone)-induced oocyte maturation in carp. Reproduction 151, 285–296.
Moniruzzaman, M., Midday, P., Dhara, A., Das, D., Ghosal, I., Mukherjee, D., Chakraborty, S.B., 2017. Change in redox state and heat shock protein expression in an Indian major carp *Cirrhinus cirrhosus* exposed to zinc and lead. J. Toxicol. Sci. 42, 731–740.
Moundipa, P.F., Beboy, N.S.E., Zelefack, F., Ngoula, S., Tsamo, E., Schill, W.-B., Monsees, T.K., 2005. Effects of *Basella alba* and *Hibiscus macranthus* extracts on testosterone production of adult rat and bull leydig cells. Asian J. Androl. 7, 411–417.
Mubarik, M.S., Amna, I., Hussain, S.M., Farhat, J., Khizar, S., Sajid, Y., Shahzad, A., Khurram, F., Khan, M.T., Salma, N., Bilal, A., 2015. Survival, growth and body composition of *Cyprinus carpio* under different levels of temperature and salinity. Int. J. Biosci. 6, 132–141.
Mukherjee, J., Moniruzzaman, M., Chakraborty, S.B., Lek, S., Ray, S., 2017a. Towards a physiological response of fishes under variable environmental conditions: an approach through neural network. Ecol. Indic. 78, 381–394.
Mukherjee, M., Moniruzzaman, M., Kumar, S., Das, D., Chakraborty, S.B., 2017b. Neuronal and oxidative damage in the catfish brain Alleviated After *Mucuna* seed extract treatment. IJPPR 9, 52–57.
Ong, J.J.L., Rountrey, A.N., Meeuwig, J.J., Newman, S.J., Zinke, J., Meekan, M.G., 2015. Contrasting environmental drivers of adult and juvenile growth in a marine fish: implications for the effects of climate change. Sci. Rep. 5, 10859.
Paital, B., Chainy, G.B.N., 2012. Effects of salinity on O2 consumption, ROS generation and oxidative stress status of gill mitochondria of the mud crab *Scylla serrata*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155, 228–237.
Pankhurst, N.W., 2011. The endocrinology of stress in fish: an environmental perspective. Gen. Comp. Endocrinol. 170, 265–275.
Pottinger, T.G., 2017. Modulation of the stress response in wild fish is associated with variation in dissolved nitrate and nitrite. Environ. Pollut. 225, 550–558.
Pratheeshkumar, P., Son, Y.O., Divya, S.P., Roy, R.V., Hitron, J.A., Wang, L., Kim, D., Dai, J., Asha, P., Zhang, Z., Wang, Y., 2014. Luteolin inhibits Cr (VI)-induced malignant cell transformation of human lung epithelial cells by targeting ROS mediated multiple cell signaling pathways. Toxicol. Appl. Pharmacol. 281, 230–241.
Pronsato, L., La Colla, A., Vasconsuelo, A., Boland, R., Milanesi, L., 2016. Effect of testosterone on the regulation of protein and gene expression related to oxidative stress damage in C2C12 cells. Bone 83, 276.
Rai, S.N., Birla, H., Zahra, W., Singh, S.S., Singh, S.P., 2017. Immunomodulation of Parkinson's disease using *Mucuna pruriens* (Mp). J. Chem. Neuroanat. 85, 27–35.
Rind, K., Beyrend, D., Blondeau-Bidet, E., Charmantier, G., Cucchi, P., Lignot, J.H., 2017. Effects of different salinities on the osmoregulatory capacity of Mediterranean sticklebacks living in freshwater. J. Zool. 303, 270–280.
Salleh, A.F.M., Amal, M.N.A., Nasruddin, N.S., Zulkifli, S.Z., Yusuff, F.M., Ibrahim, W.N.W., Ismail, A., 2017. Water pH effects on survival, reproductive performances, and ultrastructure of gonads, gills, and skins of the Javanese medaka (*Oryzias javanicus*). Turk. J. Vet. Anim. Sci. 41.
Scott, G.R., Wood, C.M., Sloman, K.A., Iftikar, F.I., De Boeck, G., Almeida-Val, V.M.F., Val, A.L., 2008. Respiratory responses to progressive hypoxia in the Amazonian oscar, *Astronotu socellatus*. Respir. Physiol. Neurobiol. 162, 109–116.
Serrano, X., Grosell, M., Serafy, J.E., 2010. Salinity selection and preference of the grey snapper *Lutjanus griseus*: field and laboratory observations. J. Fish. Biol. 76, 1592–1608.
Sharma, V.K., Sohn, M., 2009. Aquatic arsenic: toxicity, speciation, transformations, and remediation. Environ. Int. 35, 743–759.
Sies, H., 2016. The concept of oxidative stress after 30 years. In Biochemistry of Oxidative Stress. Springer, Cham, pp. 3–11.
Sinha, A.K., AbdElgawad, H., Zinta, G., Dasan, A.F., Rasoloniriana, R., Asard, H., Blust, R., Boeck, G.D., 2015. Nutritional status as the key modulator of antioxidant responses induced by high environmental ammonia and salinity stress in European sea bass (*Dicentrarchus labrax*). PLoS One 10, e0135091.
Tellis, M.S., Alsop, D., Wood, C.M., 2012. Effect of copper on the acute cortisol response and associated physiology in rainbow trout. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155, 281–289.
Tipsmark, C.K., Madsen, S.S., 2009. Distinct hormonal regulation of Na+, K+ -ATPase genes in the gill of Atlantic salmon (*Salmo salar L.*). J. Endocrinol. 203, 301–310.
Vaz, P.G., Kebreab, E., Hung, S.S., Fadel, J.G., Lee, S., Fangue, N.A., 2015. Impact of nutrition and salinity changes on biological performances of green and white sturgeon. PLoS One 10, e0122029.
Whitney, J.E., Al-Chokhachy, R., Bunnell, D.B., Caldwell, C.A., Cooke, S.J., Eliason, E.J., Rogers, M., Lynch, A.J., Paukert, C.P., 2016. Physiological basis of climate change impacts on North American inland fishes. Fisheries 41, 332–345.
Williams, L., Rota, A., 2010. Impact of climate change on fisheries and aquaculture in the developing world and opportunities for adaptation. Fisheries Thematic Paper: Tool for Project Design.
Xu, Z., Gan, L., Li, T., Xu, C., Chen, K., Wang, X., Qin, J.G., Chen, L., Li, E., 2015. Transcriptome profiling and molecular pathway analysis of genes in association with salinity adaptation in nile tilapia *Oreochromis niloticus*. PLoS One 10, e0136506.